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13. ABSTRACT (Maximum 200 Words)

In the present study, ovarian carcinoma cells (OVCA) derived from 15 patients were successfully fused with autologous DC in 10 cases and allogeneic DC in 7 cases (both autologous and allogeneic fusion cells were formed in 4 cases). The created heterokaryons expressed tumor-associated antigens, such as CA-125, MUC1 and/or HER2/neu, and DC-derived co-stimulatory and adhesion molecules. The fusion cells were functional in stimulating the proliferation of autologous T cells. In addition, $CD4^+$ and $CD8^+$ T cells derived from patients with ovarian cancer were stimulated by fusion cells to secret high level of IFN-r as demonstrated by intracellular staining in 8 patients. Significantly, T cells primed by fusion cells produced MHC class I-dependent lysis of autologous ovarian tumor cells. Furthermore, MUC1-specific CTL were generated from a HLA-A2 patient with ovarian carcinoma positive for MUC1 as demonstrated by MHC class I/MUC1 peptide tetrameric analysis. These findings indicate that fusions of human ovarian cancer cells with autologous or allogeneic DC activate both $CD4^+$ and $CD8^+$ T cells and are associated with potent and antigen-specific antitumor responses against autologous ovarian cancer cells.

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INTRODUCTION:

Ovarian cancer (OVCA) cells have been found to overexpress CA-125, HER2/neu and MUC1 tumor-associated antigens. Cell mediated immunity directed against these tumor antigens has been documented in patients with ovarian cancer (1-3). However, these responses are often muted and ineffective in eradicating the tumors. Ovarian cancer cells can evade host immune recognition by presentation of antigen in the absence of costimulatory signals or down-regulation of that particular antigen. Thus, the activation and boosting of the host's normal defense mechanisms against antigens selective for or overexpressed in ovarian cancer cells represents a potentially useful antitumor strategy.

Dendritic cells (DC) have been recognized as the most potent antigen-presenting cells. DC express MHC class I and II, costimulatory and adhesion molecules that provide secondary signals for stimulation of naïve T cells (4, 5). The fusion of DC with carcinoma cells has created a hybrid cell that expresses DC-derived MHC class I and II and costimulatory molecules as well as tumor-derived antigens and that induces a polyclonal antitumor immunity and eliminates established metastasis in animal models (6). The present study is to extend these findings to humans by fusion of patient-derived ovarian carcinoma cells with autologous or allogeneic DC. The immune responses induced by the fusion cells are determined.

BODY:

The proposed study involves the fusion of dendritic cells with patient-derived ovarian cancer cells, characterization of the fusion cells and measurement of immune responses induced by the fusion cells. In the period from August 1, 2001 to July 31, 2002, the project had been executed as planned.

Task-1. To study the feasibility of fusions of patient derived DC with ovarian cancer cells

A. Clinical sample collection. In this year, we total collected 18 samples including 15 ovarian carcinomas (OVCA) and 3 other types of cancers metastasis to ovarian organ. Ovarian cancer samples from primary and recurrent lesions were summarized in table 1.

Table 1. Samples from OVCA patients

Patient	Diagnosis	Clinical data	Samples
1	Poory differentiated serous ovary carcinoma with metastasis	Recurrent	Tumor (0.28g)
2	Adenocarcinoma	Primary	Tumor (36g)
3	Papillary serous carcinoma with metastases	Recurrent	Ascites (400ml)
4	Serous ovarian carcinoma	Primary	Tumor (340g)
5	Serous ovarian carcinoma	Primary	Tumor (46g)
6	Serous ovarian carcinoma	Recurrent	Tumor (23g) Ascities (400ml)
7	Transitional carcinoma (Ovary, grade 3/3)	Primary	Tumor (11g)
8	Serous ovarian carcinoma with metastases	Recurrent	Tumor (4.86g)
9	Papillary serous carcinoma with metastases	Recurrent	Tumor (1g)
10	Serous ovarian carcinoma	Recurrent	Tumor (3g)
11	Serous ovarian carcinoma	Primary	Tumor (18.1g)
12	Serous ovarian carcinoma	Primary	Tumor (86.7g) Ascites (950ml)
13	Serous ovarian carcinoma	Primary	Tumor (49.8g) Ascites (900ml)
14	Serous ovarian carcinoma with metastases	Recurrent	Tumor (2.6g) Pleural fluid (80ml)
15	Serous ovarian carcinoma	Primary	Tumor (1g) Ascites (14ml)

B. Tumor cell isolation. In 15 patients diagnosed as ovarian cancer, we obtained 9 solid lesions, 1 ascites and 5 solid lesions as well as metastatic fluids. We successfully isolated tumor cells from both the solid lesions and metastatic ascites in 14 cases and their phenotypes were determined. The cancer cells expressed CA-125, MUC1 and Her-2neu tumor antigens. Table 2 and 3 show the numbers of tumor cells isolated from solid lesions or metastatic fluids, respectively, and their antigen expression.

Table 2. Isolation of OVCA cells from solid tumors in 5 patients

Patients	Diagnosis	Tumor samples	Weight of tumor	Tumor cells	Purity (%)	Antigen expression
11	Serous ovarian carcinoma	Tumor tissue	18.1g	$5.5x10^6$	90%	OC-125, MUC1 MHC class I
12	Serous ovarian carcinoma	Tumor tissue	86.7g	$3.4x10^7$	92.8%	OC-125, MUC1 MHC class I
13	Serous ovarian carcinoma	Tumor tissue	49.8g	$8x10^6$	85%	OC-125, MUC1 MHC class I
14	Serous ovarian carcinoma with metastases	Tumor tissue	2.6g	$7.5x10^6$	94%	OC-125, MUC1 MHC class
15	Serous ovarian carcinoma	Tumor tissue	1g	$1x10^7$	89%	OC-125, MUC1 MHC class

Table 3. Isolation of OVCA cells from metastatic fluids in 5 patients

Patients	Diagnosis	Tumor samples	Weight of tumor	Tumor cells	Purity (%)	Antigen expression
3	Papillary serous carcinoma with metastases	Ascites	400 ml	4×10^6	90%	OC-125, MUC1 MHC class I
12	Serous ovarian carcinoma	Ascites	950 ml	20.2×10^7	89%	OC-125, MUC1 MHC class I
13	Serous ovarian carcinoma	Ascites	900ml	1.5×10^6	80.1%	OC-125, MUC1 MHC class I
14	Serous ovarian carcinoma with metastases	Pleural fluid	80ml	1.75×10^7	90%	OC-125, MUC1 MHC class I
15	Serous ovarian carcinoma	Ascites	14ml	3×10^6	85%	OC-125, MUC1 MHC class I

C. Dendritic cells (DC) isolation. DC were generated from peripheral blood of 10 OVCA patients. We failed to generate autologous DC in 3 patients due to recent chemotherapy (patient #6, #13 and #14). Allogeneic DC were generated from leukopacks (healthy donor) of 7 individuals and fused with patient's OVCA including the 3 patients without the autologous DC. The DC expressed MHC class I and II, costimulatory and adhesion molecules.

D. Fusions of autologous and allogenic DC to OVCA cells. OVCA cells were successfully fused to autologous (auto-FC) or allogeneic (allo-FC) DC , respectively, to create autologous (DC and OVCA cells from the same patient) or allogeneic (patient-derived OVCA cells fused to DC from healthy donor) fusion cells in 13 cases.

Task 2. To characterize the fusion cells from patient derived DC with ovarian cancer cells.

A. The fusion cells were characterized by flow cytometric analysis and immunohistochemical staining. The fusion cells expressed CA-125 and MUC1 tumor associated antigens, as well as DC-derived MHC class II, B7-1 and B7-2. Figure 1 shows the antigen expression of OVCA, DC or DC/OVCA fusion cells with immunohistochemical staining.

OVCA DC FC/OVCA

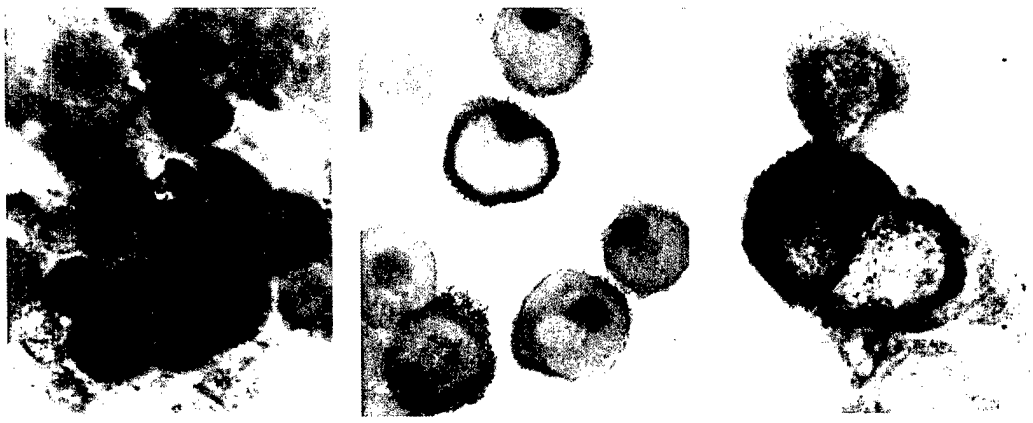


Fig.1. Antigen expression of OVCA, DC or DC/OVCA fusion cells with immunohistochemical staining. Patient-derived OVCA, DC and fusion cells (FC/OVCA) were stained with monoclonal antibodies against MUC1, MHC class II and examined under microscopy (Magnification x40). Note that fusion cells stained positive for both MUC1 (red color) and MHC class II (blue color).

B. The frequency of fusion was determined with double immunohistochemical staining and flow cytometry analysis. Table 4 shows the frequency of fusions in 4 cases.

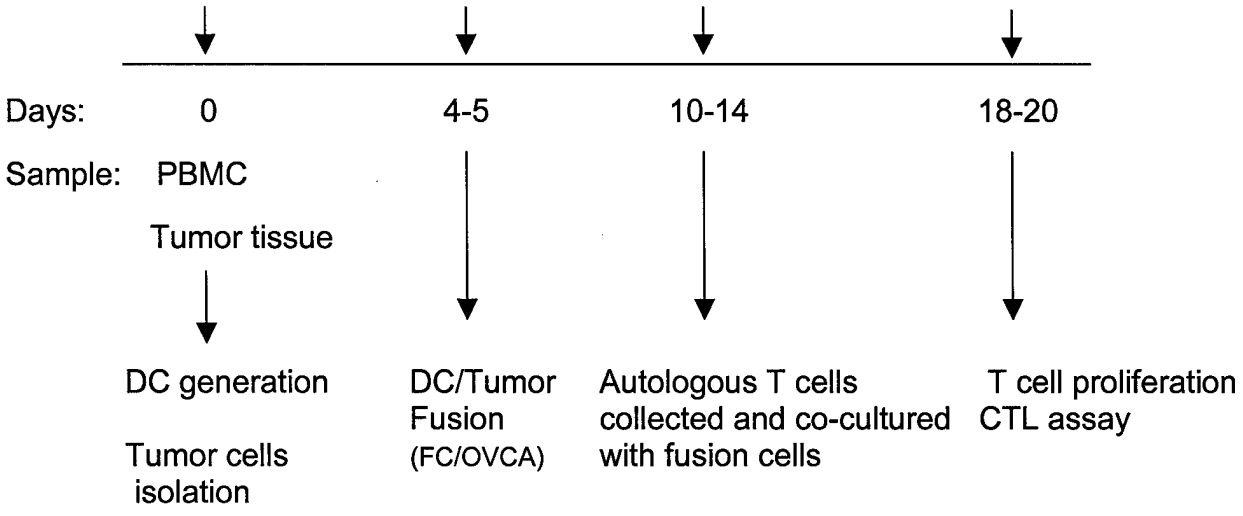
Table 4. The frequency of DC/tumor fusions by flow cytometry analysis

#	Antibody Staining	Anti-HLA-DR Anti-MUC1	Anti-HLA-DR Anti-OC125	Anti-CD86 Anti-OC125	Anti-CD83 Anti-OC125
1	Single	MUC1 (23%)	OC-125 (36%)	CD86 (73%)	CD83 (27%)
2	Double	28.5%	32.45%	ND	ND
3	Double	ND	34.74%	ND	ND
4	Double	29.9%	32.62%	30.0%	10.84%

Task 3. To determine whether the patient derived DC-ovarian cancer fusion cells will induce and expand autologous tumor specific CTLs in vitro

The immune responses induced by the fusion cells were determined by coculture of fusion cells with patient-derived peripheral blood monocytes (PBMC). Figure 2 is a diagram to illustrate the experiment. The nonadherent population of autologous PBMC were cocultured with either fusion cells or DC for 7-10 days. Then the T cells were isolated with nylon wool, and analyzed for CTL activity with ^{51}Cr-release assay.

Fig. 2. The schedule of DC-OVCA fusions and T cells stimulation



A. T cell proliferation stimulated by fusion cells. The ability to stimulate T cell proliferation by fusion cells was assessed with standard ^{3}H-thymidine uptake assay. Table 5 and 6 show both autologous and allogeneic fusion cells, but not OVCA cells, stimulated T cell proliferation (Table 5 and 6). In addition, we compared the T cell proliferation stimulated by auto-FC or allo-FC in 4 patients when both auto-FC and allo-FC were available (Table 7). Allo-FC were more potent stimulators of T cell proliferation.

Table 5. T cell proliferation stimulated by autologous DC/tumor fusion cell

Patient	Autologous T cells stimulated by auto-FC	Autologous T cells stimulated by OVCA cells
3	22329 ± 3310	3474 ±1640
5	6520 ± 1395	1695 ± 392
9	2126 ± 140	164 ± 37
11	478 ± 123	142 ± 99
15	4712 ± 1260	395 ± 28

Table 6. T cell proliferation stimulated by allogenic DC/tumor fusion cells

Patient	Patient's T cells stimulated by allo-FC	Patient's T cells stimulated by autologous OVCA cells
3	28003 ± 1096	3431 ± 1334
5	25128 ± 5634	2683 ± 818
9	40025 ± 4373	122 ± 25
10	35026 ± 2534	6359 ± 1210
11	51719 ± 14520	1062 ± 813
13	74033 ± 4672	4381 ± 417
14	19721 ± 3085	846 ± 236

Table 7. Comparison of allo-FC and auto-FC in T cell proliferation

Patient	Patient's T cells stimulated by auto-FC/OVCA	Patient's T cells stimulated by allo-FC/OVCA
3	22329 ± 3310	28003 ± 1096
5	6520 ± 1395	25128 ± 5634
9	2126 ± 140	40025 ± 4373
11	478 ± 123	51719 ± 14520

B. Cytokine production of T cells. Cytokine production was measured by intracellular staining. Both patient-derived CD4 and CD8 T cells stimulated by fusion cells produced higher level of IFN-γ in 8 patients (Table 8).

Table 8. Cytokine production of T cells induced by DC/tumor fusion cells

Patient	**IFN-γ** secreting (%) in CD4-T cells	**IFN-γ** secreting (%) in CD8-T cells	**IL-4** secreting (%) in CD4-T cells	**IL-4** secreting (%) in CD8-T cells
3	2.1	1.18	0.36	0.16
5	9.08	10.88	1.32	1.22
6	3.92	4.92	2.66	1.8
11	40.9	10.17	*ND*	*ND*
12	14.8	6.62	1.34	0.56
13	8.32	6.48	0.6	0.32
14	1.98	6.46	0.34	0.2
15	1.46	26.84	1.31	0.31

C. Cytotoxic activity. CTL activity induced by autologous or allogeneic DC/tumor fusion cells against autologous OVCA cells was measured using standard ^{51}Chromium-release assay. Table 9 summarizes the CTL activity of 10 patients induced by autologous FC/OVCA fusion cells against autologous OVCA targets at 60:1 effector:target ratio. The CTL activity induced by allo-FC against OVCA cells was also measured in 3 patients from them we failed to generate autologous DC due to recent chemotherapy.

Table 9. CTL activity induced by auto-FC or allo-FC against autologous OVCA cells

Diagnosis	Tumor cell origin	Patients	DC/tumor fusions	(%) CTL activity against Autologous OVCA cells
OVCA	Primary	2, 5, 7, 11, 12, 15.	Autologous FC/OVCA fusions	63.4, 53.4, 59.02, 43.37, 49.2, 67.59
OVCA	Recurrent	1, 3, 8, 9.	Autologous FC/OVCA fusions	71.3, 50.42, 43.24, 98.8
OVCA	Primary& Recurrent	13, 6, 14.	Allogeneic-DC & OVCA fusions	35.54, 39.38, 25.99

Figure 3 shows the comparison of CTL activity induced by auto-FC and allo-FC against autologuos OVCA targets in 4 patients when both fusion cells were available. Auto-FC induced higher CTL activity against OVCA cells than allo-FC.

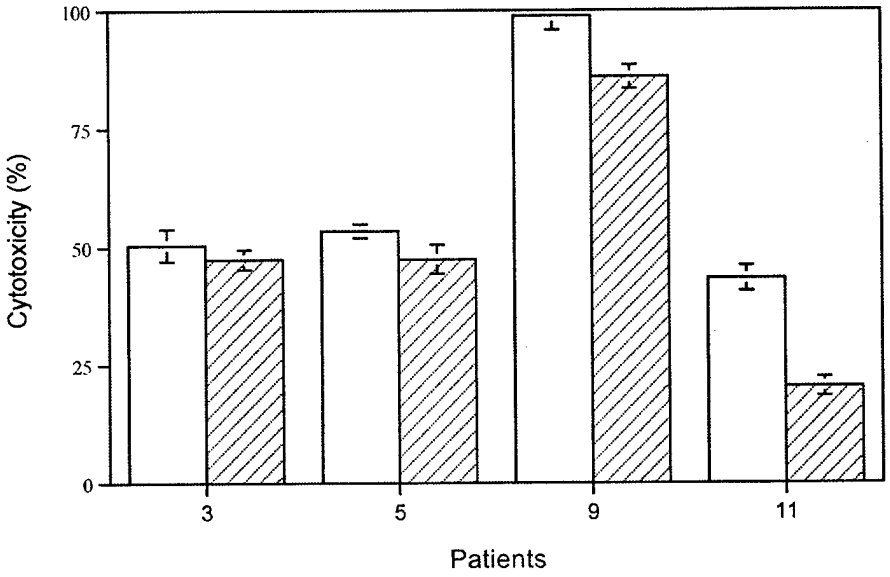


Fig. 3. Comparison of CTL activity induced by auto-FC and allo-FC against autologous OVCA cells in 4 patients. Autologous T cells stimulated by auto-FC (gray bar) or allo-FC (hatch bar) against autologous OVCA targets at 60:1 effector:target ratio.

***D*. Fusion of OVCA cells with HLA matched DC**. HLA-A2 patient-derived OVCA cells were fused to DC positive for HLA-A2. The HLA-matched fusion cells were cocultured with patient-derived T cells. The cytokine production by the T cells was measured and the frequency of MUC1-specific CTL was determined by MHC class I/MUC1 peptide tetramer (Figure 4).

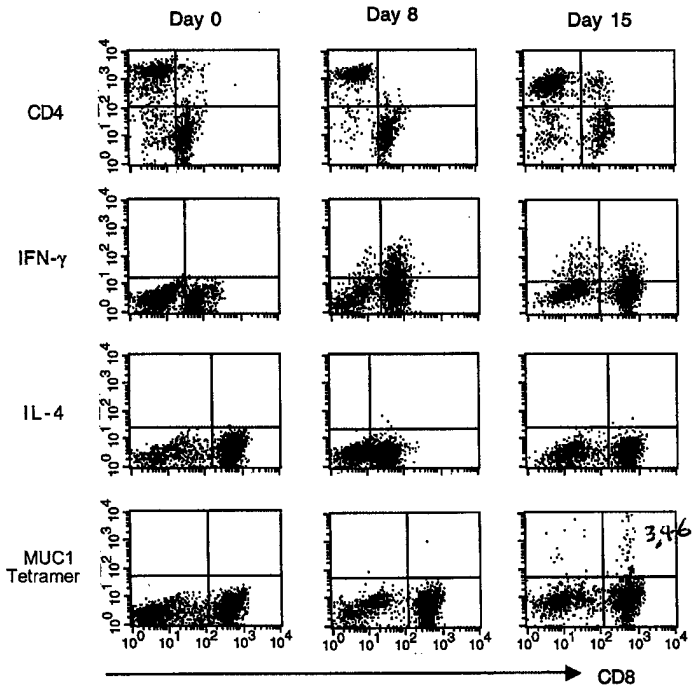


Fig. 4. Phenotype and tetrameric analysis of T cells induced by fusion cells. Patient-derived PBMC were cocultured with fusion cells for 5 days, stained with monoclonal antibodies against CD4, CD8, IFN-r, IL-4 or MHC class I/MUC1 peptide tetramer and analyzed by flow cytometry.

E. Antigen-specificity and MHC restriction of CTL. Autologous T cells induced by fusion cells lysed autologous OVCA, but not autologous monocytes, MCF-7 and BT-20 breast cancer cells, OV-3 ovarian cancer cells or NK sensitive-K562 target cells (Figure 5). The CTL activity was blocked by MHC class I monoclonal antibody indicating the lysis is MHC I dependent and antigen specific (Figure 5).

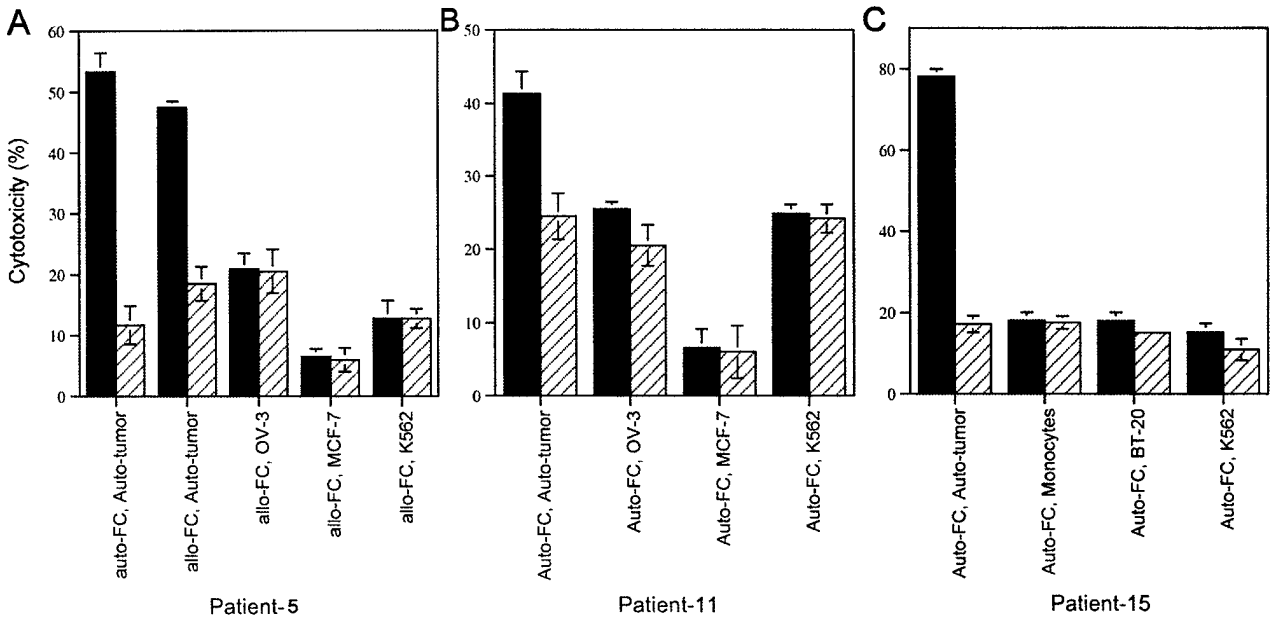


Fig. 5. Specificity of CTL activity. T cells from 3 ovarian cancer patients (A-C) were cocultured with FC/OVCA for 5 days. The T cells were purified with nylon wool and cocultured with ^{51}Cr-labeled autologous OVCA, autologous monocytes (MC), MCF-7 and BT-20 breast cancer cells, OV-3 ovarian cancer cell or K562 cells at 30:1 ratio (solid bar). The targets were also pre-incubated with an anti-MHC class I mAb (W6/32, 1:100 dilution) and then CTL activity was measured (hatched bar). Percentage of cytotoxicity was determined by ^{51}Cr release. The results were expressed as the mean ± SD of three replicates.

KEY RESEARCH ACCOMPLISHMENT:

- Ovarian cancer cells were isolated from patients and fused with either autologous or allogeneic DC.
- Fusion cells expressed MHC class I and class II, co-stimulatory, and ICAM molecules as well as tumor-derived antigens.
- Both allogeneic and autologous DC fused with patients-derived OVCA efficiently stimulated the patient's T cell proliferation.
- Antigen-specific and MHC class I-restricted CTL against autologous OVCA cells were induced by fusion cells.
- Allogeneic fusion cells are more potent activator of T cell proliferation whereas autologoous and HLA-A2 matched DC/OVCA fusion cells induced higher CTL activity against autologous OVCA cells.

- Higher level of cytokine was produced by T cells induced by fusion cells.
- MUC1-specific CD8 T cells were activated by fusion cells as demonstrated by MHC class I/MUC1 peptide tetrameric analysis.

REPORTABLE OUTCOMES:

1. Gong J., Nikrui N., Chen D., Koido S., Wu Z., Tanaka Y., Cannistra S., Avigan D. and Kufe D. Fusion of Human Ovarian Carcinoma Cell with Autologous and Allogeneic Dendritic cells Induce Anti-Tumor Immunity. J. Immunol. 2000, 165:1705-1711.
2. Shigeo Koido, Najmosama Nikrui, Zhinan Xia and Jianlin Gong. Induction of $CD4^+$ and $CD8^+$ T cells from ovarian cancer patients by fusion cells associated with lysis of autologous tumor cells (AACR meeting, 2002 April).

Conclusions:

DC and ovarian carcinoma cells were isolated from primary and metastatic lesions of 15 patients with ovarian cancer. The OVCA cells were fused to autologous DC in 10 cases and allogeneic DC in 7 cases (both allo-FC and auto-FC were formed in 4 cases). The fusion cells expressed MHC class I and II, costimulatory molecules and tumor-derived antigens. Unlike the OVCA cells, both autologous and allogeneic fusion cells stimulated autologuos T cell proliferation and IFN-γ production by T cells. More importantly, autologous CTL were induced by the fusion cells to lyse patient-derived OVCA cells. The CTL activity were antigen-specific and MHC-restricted. Collectively, patient-derived OVCA cells can be fused to either autologous or allogeneic DC. The benefit of allogeneic fusion is the unlimited source of DC. In addition, T cells against allo-antigens can be stimulated. In terms of T cell proliferation, allo-FC are more potent than auto-FC. However, auto-FC induced higher level of CTL activity compared with those of allo-FC. These results indicate the potential application of either autologous or allogeneic fusion cells as vaccine in clinical setting.

In the coming year, we will focus on the development of DC/tumor fusion cells as a vaccine for patient use. For example, it is necessary to freeze and irradiate the fusion cells if they are used as vaccine for patient. Therefore, the impact of freezing and irradiation on the efficacy of fusion cells should be determined. In addition, we will also design a protocol for phase I clinical trial of fusion cells for patient with ovarian cancer.

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6. Gong, J., Chen, D., Kashiwaba, M., and Kufe, D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med, *3:* 558-561., 1997.

APPENDICES:

- Paper

Gong J., Nikrui N., Chen D., Koido S., Wu Z., Tanaka Y., Cannistra S., Avigan D. and Kufe D. Fusion of Human Ovarian Carcinoma Cell with Autologous and Allogenic Dendritic cells Induce Anti-Tumor Immunity. J. Immunol. 2000, 165:1705-1711.

- Abstract:

Shigeo Koido, Najmosama Nikrui, Zhinan Xia and Jianlin Gong. Induction of $CD4^+$ and $CD8^+$ T cells from ovarian cancer patients by fusion cells associated with lysis of autologous tumor cells (AACR meeting-Abstract, 2002 April).

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INDUCTION OF CD4+ AND CD8+ T CELLS FROM OVARIAN CANCER PATIENTS BY FUSION CELLS ASSOCIATED WITH LYSIS OF AUTOLOGOUS TUMOR CELLS

Shigeo Koido, Najmosama Nikrui, Zhinan Xia, Jianlin Gong
Dana Farber Cancer Institute, Boston MA, Mass. general hospital, Harvard Medical School, Boston MA and Beth Israel Deaconess Medical Center, Boston MA

Dendritic/tumor fusion cell vaccine has been developed in our lab. The antitumor immunity induced by fusion cells has been demonstrated in murine models and in humans. In the present study, ovarian carcinoma cells (OVCA) derived from 8 out of 9 patients were successfully fused with autologous DC. The created heterokaryons expressed tumor-associated antigens, such as CA-125, MUC1 or/and HER2/neu, and DC-derived co-stimulatory and adhesion molecules. The fusion cells were functional in stimulating the proliferation of autologous T cells. The level of T cell proliferation was increased six-seven folds when cocultured with fusion cells. Significantly, CD4+/CD8+ T cells derived from patients with ovarian cancer were stimulated by fusion cells to secret high level of IFN-r as demonstrated by intracellular staining in 7 patients. The T cells primed by fusion cells produced MHC class I-dependent lysis of autologous ovarian tumor cells. Furthermore, MUC1-specific CTL were generated from a

Fusions of Human Ovarian Carcinoma Cells with Autologous or Allogeneic Dendritic Cells Induce Antitumor Immunity[1]

Jianlin Gong,[2]* Najmosama Nikrui,† Dongshu Chen,* Shigeo Koido,* Zekui Wu,* Yasuhiro Tanaka,* Stephen Cannistra,‡ David Avigan,‡ and Donald Kufe*

Human ovarian carcinomas express the CA-125, HER2/neu, and MUC1 tumor-associated Ags as potential targets for the induction of active specific immunotherapy. In the present studies, human ovarian cancer cells were fused to human dendritic cells (DC) as an alternative strategy to induce immunity against known and unidentified tumor Ags. Fusions of ovarian cancer cells to autologous DC resulted in the formation of heterokaryons that express the CA-125 Ag and DC-derived costimulatory and adhesion molecules. Similar findings were obtained with ovarian cancer cells fused to allogeneic DC. The fusion cells were functional in stimulating the proliferation of autologous T cells. The results also demonstrate that fusions of ovarian cancer cells to autologous or allogeneic DC induce cytolytic T cell activity and lysis of autologous tumor cells by a MHC class I-restricted mechanism. These findings demonstrate that fusions of ovarian carcinoma cells and DC activate T cell responses against autologous tumor and that the fusions are functional when generated with either autologous or allogeneic DC. ***The Journal of Immunology,*** **2000, 165: 1705–1711.**

Epithelial ovarian cancer is the leading cause of death for patients with gynecologic malignancies (1, 2). The majority of women with ovarian cancer present with advanced stage disease. Moreover, most patients with advanced disease recur after standard treatment with surgical debulking and combination chemotherapy (3). Immune-based therapies for ovarian cancer have been proposed as alternative approaches to improve survival (2, 4). In this context, ovarian cancer cells express mutated forms of the p53 (5–7) and/or BRCA-1 (8, 9) tumor suppressor genes. Ovarian tumors also overexpress the CA-125 (10–12) and DF3/MUC1 (13) carcinoma-associated Ags. In addition, these tumors overexpress the HER2/neu (c-erB2) and epidermal growth factor receptors (14–17). Thus, certain targets for immunotherapy of ovarian cancer are already known, and others, although they remain undefined, presumably exist.

Dendritic cells (DC)[3] are potent APC that can elicit primary immune responses (18). DC express MHC class I and II, costimulatory and adhesion molecules that provide secondary signals for stimulation of naive T cell populations (19, 20). In animal models, antitumor vaccines have been developed by pulsing DC with peptides derived from tumor Ags (21, 22). With regard to ovarian cancer, human DC loaded with HER2/neu peptides have been shown to stimulate proliferation of autologous T cells that induce lysis of peptide-pulsed targets (23, 24). Other studies have demonstrated that transduction of DC with viral vectors, which encode tumor Ags, confers presentation of tumor peptides to T cells (25–27). Using this strategy, DC transduced to express the MUC1 Ag have been shown to induce anti-MUC1 immune responses (25, 28). However, immunotherapeutic approaches that are dependent on the response to a single Ag are potentially subject to resistance by down-regulation of that Ag.

Other DC-based strategies have been developed to induce a polyclonal immune response against multiple tumor Ags. DC have been pulsed with tumor cell lysates (29–31), loaded with peptides acid-eluted from tumor cells (32, 33), and transfected with tumor cell RNA (34, 35). Fusions of DC and tumor cells have also been developed to induce a polyclonal antitumor immune response (36). In this approach, multiple tumor Ags, including those yet unidentified, are endogenously processed and presented by MHC class I pathways (36). Vaccination with fusions of murine tumor cells and syngeneic DC have been shown to eliminate established metastatic disease (36–39). Moreover, immunization of MUC1-transgenic mice with MUC1-positive fusion cells reversed immunologic unresponsiveness to MUC1 and induced immunity against MUC1-expressing tumors (40).

The present studies demonstrate the fusion of human ovarian carcinoma cells with autologous and allogeneic DC. We show that the human ovarian/DC fusions express both ovarian carcinoma-associated Ags and DC-derived MHC class II and costimulatory molecules. The fusion cells stimulate autologous T cells and induce CTL activity against autologous ovarian tumor cells.

Materials and Methods

Isolation of PBMC

Mononuclear cells were isolated from the peripheral blood of patients with ovarian cancer and normal donors by Ficoll-Hypaque density gradient centrifugation. The PBMC were cultured in RPMI 1640 medium containing 1% autologous serum for 1 h. The nonadherent cells were removed, and the T cells were purified by nylon wool separation. The adherent cells were cultured for 1 wk in RPMI 1640 medium containing 1% autologous serum, 1000 U/ml GM-CSF (Genzyme, Cambridge, MA), and 500 U/ml IL-4 (Genzyme). DC were harvested from the nonadherent and loosely adherent cells. The firmly adherent monocytes were cultured in RPMI 1640 medium

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[3] Abbreviations used in this paper: DC, dendritic cell; OVCA, ovarian carcinoma; h, human; MC, monocyte; OVCA/FC, fusion of the OVCA cells to autologous DC; MCF-7, breast adenocarcinoma cell line.

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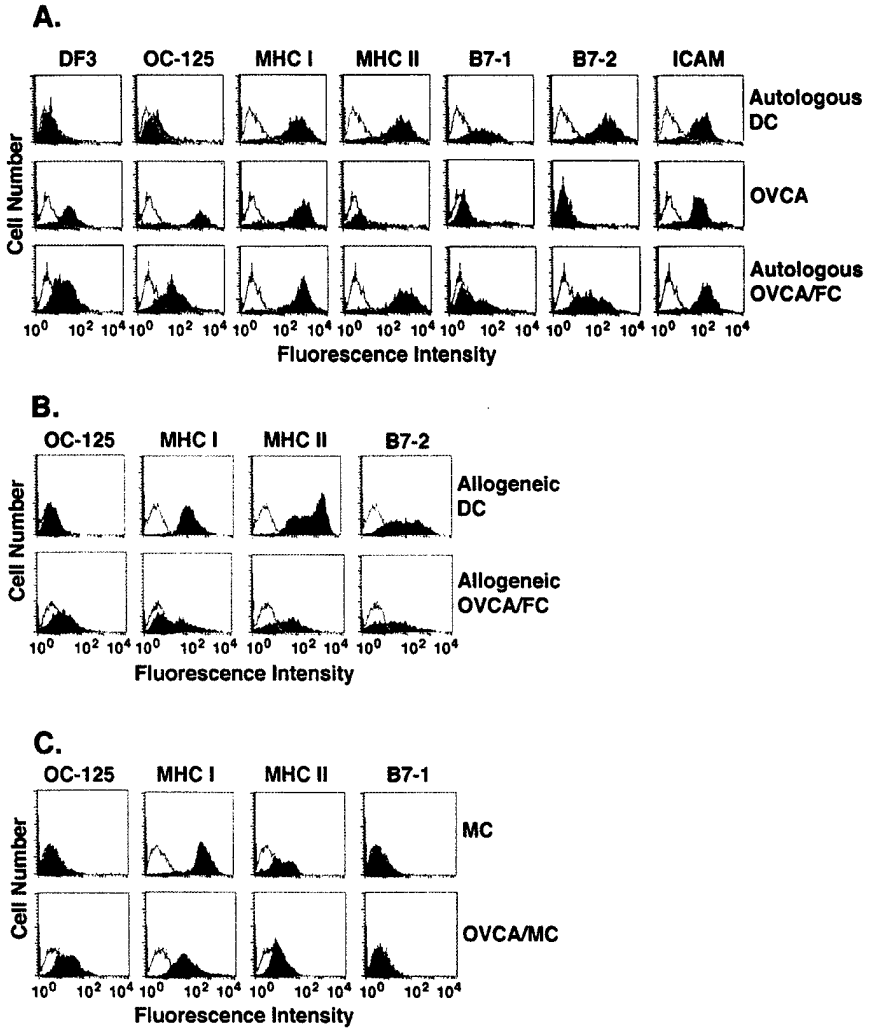

FIGURE 1. Characterization of OVCA cells fused to autologous and allogeneic DC. *A*, Patient-derived DC, OVCA cells, and DC-OVCA fusion cells (autologous OVCA/FC). *B*, DC from normal volunteers (allogeneic DC) were fused with patient-derived OVCA cells (allogeneic OVCA/FC). *C*, MC were fused with autologous OVCA cells (OVCA/MC). Cells were analyzed by flow cytometry for expression of the indicated Ags (solid area).

containing 10% autologous serum without GM-CSF/IL-4 and harvested after treatment with trypsin.

Preparation and fusion of ovarian carcinoma cells

Ovarian carcinoma (OVCA) cells obtained from primary tumors and malignant ascites were separated in HBSS (Ca^{2+}/Mg^{2+} free) containing 1 mg/ml collagenase, 0.1 mg/ml hyaluronidase, and 1 mg/ml DNase. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated autologous human serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin until fusion. Autologous or allogeneic DC were incubated with the OVCA cells for 5 min at a ratio of 10:1 in serum-free RPMI 1640 medium containing 50% polyethylene glycol. RPMI 1640 medium was then added slowly to dilute the polyethylene glycol. After washing, the cells were resuspended in RPMI 1640 medium supplemented with 10% autologous serum and 500 U/ml GM-CSF for 7–14 days.

Phenotype analysis

Cells were incubated with mouse anti-human Abs directed against DF3/MUC1 (mAb DF3) (13), CA-125 (mAb OC-125) (41), MHC class I (W6/32), MHC class II (HLA-DR), B7-1 (CD80), B7-2 (CD86), ICAM (CD54; PharMingen, San Diego, CA), and CD83 (PharMingen) for 1 h on ice. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-mouse IgG for 30 min. For dual expression analysis, cells were incubated with mAb OC-125, washed, and then incubated with phycoerythrin-conjugated anti-MHC class II, anti-B7-2, or anti-CD83 for 1 h at 4°C. Samples were washed, fixed in 2% paraformaldehyde, and analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Immunohistochemical staining

Cytospin cell preparations were fixed in acetone and incubated with mAb OC-125 for 30 min at room temperature. The slides were washed and incubated with biotinylated horse anti-mouse IgG for an additional 30 min. Staining (red color) was generated with ABC solution (Vector Laboratories, Burlingame, CA). The slides were then incubated with murine anti-human MHC class II for 30 min and then with alkaline phosphatase-labeled anti-mouse IgG. AP-ABC solution (Vector Laboratories) was used to generate a blue counterstain.

T cell proliferation assays

Cells were exposed to 30 Gy ionizing radiation and added to T cells in 96-well flat-bottom plates for 5 days. Uptake of [^{3}H]thymidine by the T cells was measured after incubation in the presence of 1 μCi/well for 12 h.

Cytotoxicity assays

T cells were stimulated with the indicated cell preparations for 1 wk in the presence of 20 U/ml human IL-2 (hIL-2). The T cells were harvested by nylon wool separation and used as effector cells in CTL assays. Autologous OVCA cells, allogeneic OVCA cells, autologous monocytes, MCF-7 breast carcinoma cells, and K562 cells were labeled with ^{51}Cr for 60 min at 37°C. After washing, targets (2×10^4) were cultured with the T cells for 5 h at 37°C. In certain experiments, the labeled target cells were incubated with mAb W6/32 (anti-MHC class I) for 30 min at 37°C before addition of the effector cells. Supernatants were assayed for ^{51}Cr release in a gamma counter. Spontaneous release of ^{51}Cr was assessed by incubation of the targets in the absence of effectors. Maximum or total release of ^{51}Cr was determined by incubation of the targets in 0.1% Triton X-100. Percentage

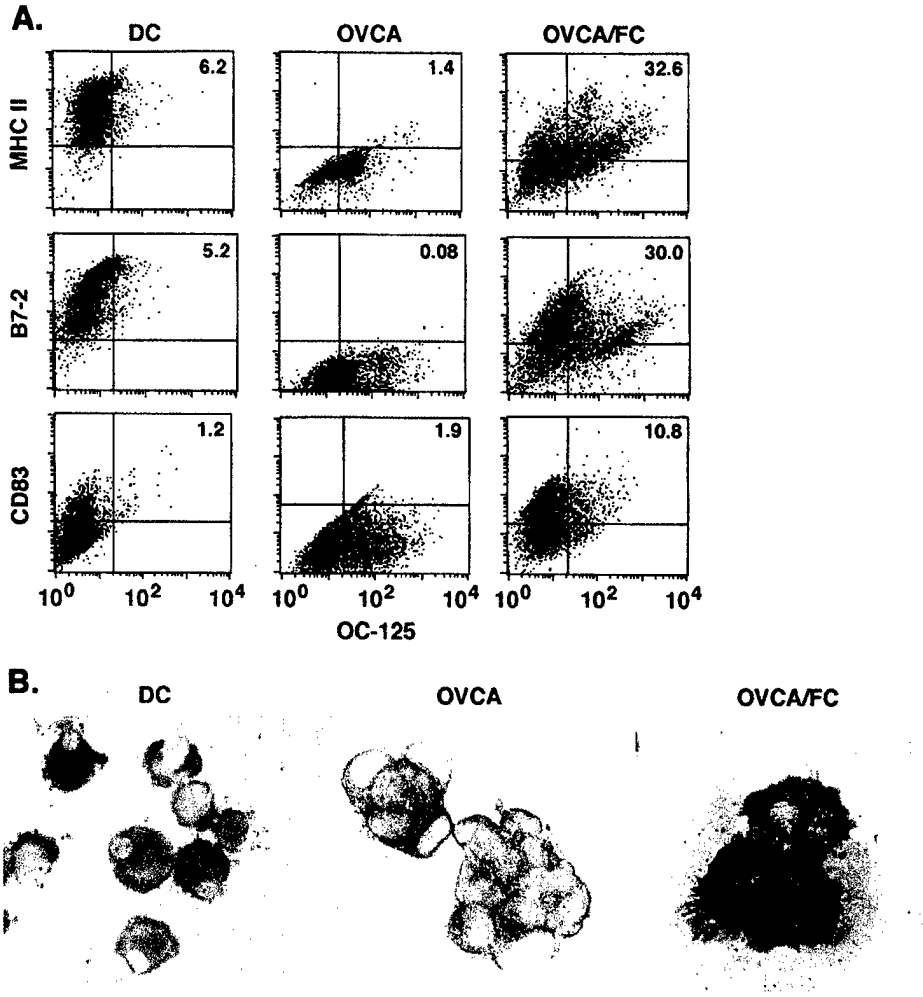

FIGURE 2. Phenotype of ovarian carcinoma cells fused to autologous DC. *A*, Autologous DC, OVCA cells, and OVCA fused with autologous DC were analyzed by bi-dimensional flow cytometry for expression of CA-125 and MHC class II, B7-2, or CD83. *B*, Cytocentrifuge preparations of DC, OVCA, and OVCA/FC cells were dual-stained with mAb HLA-DR (anti-MHC class II; blue color) and mAb OC-125 (anti-CA-125; red color). Magnification, ×40.

of specific ^{51}Cr release was determined by the following equation: percent specific release = [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

Results

Characterization of OVCA cells fused with autologous and allogeneic DC

DC were generated from patients with metastatic ovarian cancer and from normal volunteers. Adherent cells were isolated from PBMC and cultured in the presence of GM-CSF and IL-4 for 1 wk. The resulting population was subjected to FACS analysis. The DC displayed a characteristic phenotype with expression of MHC class I and class II, costimulatory molecules, and ICAM, but not of the DF3/MUC1 or CA-125 carcinoma-associated Ags (Fig. 1*A*). By contrast, OVCA cells isolated from a patient with metastatic ovarian cancer expressed MUC1, CA-125, MHC class I, and ICAM, but not MHC class II, B7-1, or B7-2 (Fig. 1*A*). Similar findings were obtained with OVCA cells obtained from primary ovarian tumors and from malignant ascites (data not shown). Fusion of the OVCA cells to autologous DC (OVCA/FC) resulted in the generation of heterokaryons that express the CA-125 and MUC1 Ags, MHC class II, B7-1, and B7-2 (Fig. 1*A*). Moreover, the pattern of Ag expression was similar when the OVCA cells were fused to allogeneic DC (Fig. 1*B*). Whereas cytokines produced by fusing OVCA cells and DC could alter expression of DC-derived molecules, the OVCA/FC exhibited similar levels of MHC class II and costimulatory molecules as found on unfused DC. As a control, monocytes (MC) were fused with autologous OVCA cells. Fusions of MC to OVCA cells also expressed CA-125 and MHC class I. However, compared with OVCA/FC, the OVCA/MC exhibited lower levels of MHC class II and little if any B7-1 (Fig. 1*C*).

Double immunofluorescence was used to assess efficiency of the fusions. In contrast to DC, the OVCA cells expressed CA-125, but not MHC class II, B7-2, or CD83 (Fig. 2*A*). Analysis of OVCA cells fused with autologous DC demonstrated that 32.6% of the population expressed both CA-125 and MHC class II (Fig. 2*A*). Assessment of CA-125 and B7-2 demonstrated that 30% of the autologous OVCA/FC expressed both Ags (Fig. 2*A*). Moreover, 10.8% of the autologous OVCA/FC population expressed both CA-125 and CD83 (Fig. 2*A*). Notably, expression of CD83 varied on DC preparations from different individuals. Immunostaining confirmed that the DC expressed MHC class II and not CA-125 (Fig. 2*B*). Conversely, the OVCA cells expressed CA-125 and not MHC class II (Fig. 2*B*). Analysis of the fusion cells (OVCA/FC) demonstrated expression of both Ags (Fig. 2*B*). These findings demonstrate the formation of heterokaryons by fusing OVCA cells to DC.

Stimulation of antitumor CTL by autologous OVCA/FC

To assess the function of OVCA/FC, the fusion cells were cocultured with autologous PBMC. As a control, the PBMC were also cultured with autologous OVCA cells. The fusion cells, but not the tumor cells, stimulated the formation of T cell clusters (Fig. 3*A*).

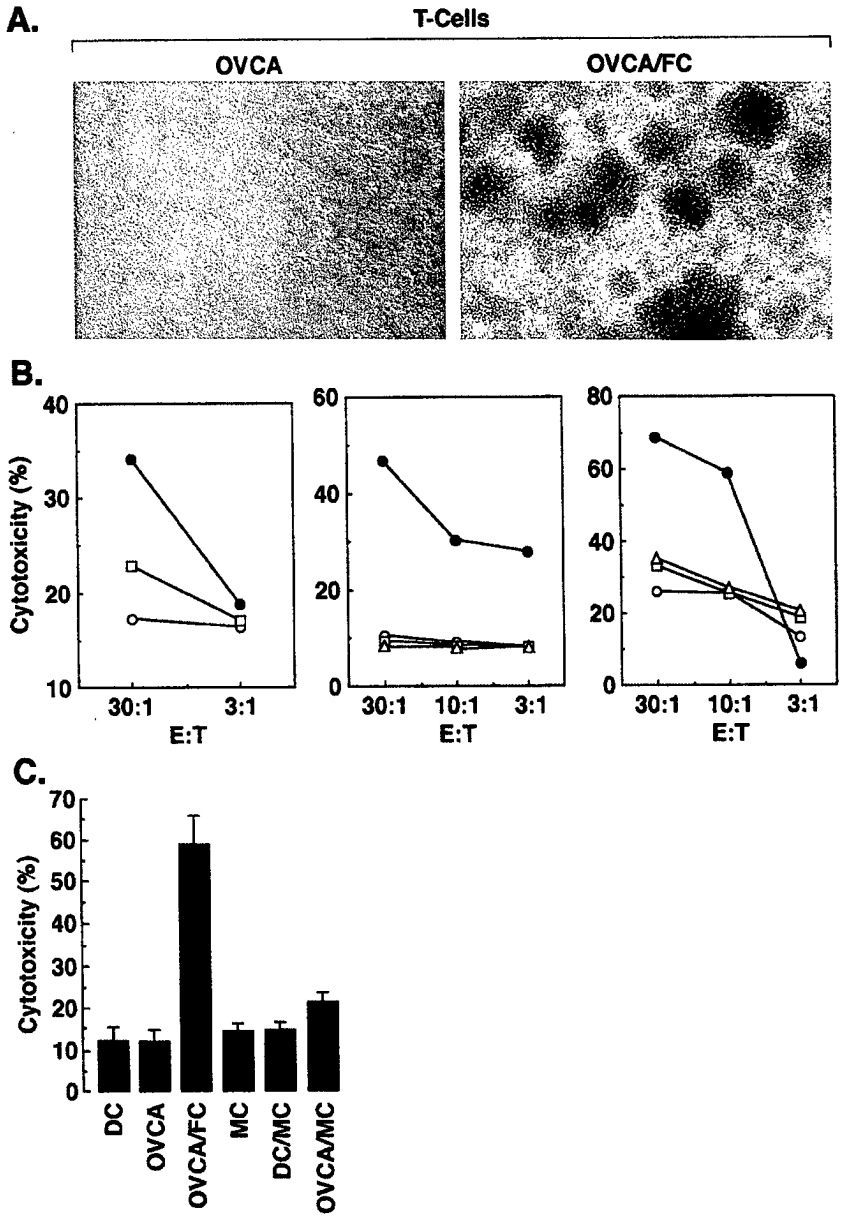

FIGURE 3. Stimulation of antitumor CTL cells by autologous OVCA/FC. *A*, PBMC were incubated with autologous OVCA cells (*left panel*) or fusion cells (OVCA/FC; *right panel*) at a ratio of 10:1 for 7 days in the presence of 20 U/ml hIL-2. Incubation with OVCA/FC cells, but not with OVCA cells, resulted in the formation of T cell clusters. *B*, T cells derived from three different patients (data from each patient are displayed in the individual panels) were cocultured with DC (○), OVCA cells (□), autologous OVCA cells mixed with DC (△), or OVCA/FC (●) for 10 days. The stimulated T cells were incubated with ^{51}Cr-labeled autologous OVCA at the indicated ratios. Percentage cytotoxicity was determined by ^{51}Cr release. *C*, CTL activity induced by OVCA/FC is compared with that obtained using DC or MC alone, with OVCA fused to MC or DC fused to MC. Percentage cytotoxicity (mean ± SD of three replicates) was determined by ^{51}Cr release.

After 10 days of stimulation, the T cells were isolated for assessment of cytolytic activity. Using autologous OVCA cells as targets, there was a low level of lysis when assaying T cells that had been incubated with autologous DC, autologous tumor, or a mixture of unfused DC and tumor (Fig. 3*B*). By contrast, T cells stimulated with the OVCA/FC were effective in inducing lysis of autologous tumor targets (Fig. 3*B*). Similar results were obtained with T cells from three patients with ovarian cancer (Fig. 3*B*). As a control, T cells stimulated with OVCA cells fused to autologous monocytes or DC fused to monocytes had little effect on stimulation of antitumor CTL activity (Fig. 3*C*).

Generation of antitumor CTL by OVCA cells fused to allogeneic DC

To assess OVCA/FC function when the fusion is performed with allogeneic DC, we stimulated autologous PBMC with OVCA cells fused to autologous or allogeneic DC. As controls, the autologous PBMC were also stimulated with unfused DC or OVCA cells. Incubation of the T cells with allogeneic DC was associated with greater stimulation than that obtained with autologous DC (Fig. 4*A*). The results also demonstrate that T cell proliferation is stimulated to a greater extent by OVCA fused to allogeneic, as compared with autologous, DC (Fig. 4*A*). Similar findings were obtained with T cells obtained from two patients (Fig. 4*A*). After stimulation for 10 days, the T cells were isolated and assessed for lysis of autologous tumor. Stimulation with unfused allogeneic or autologous DC had little if any effect on lytic function compared with that obtained with T cells stimulated in the presence of OVCA cells (Fig. 4*B*). By contrast, T cells stimulated with OVCA cells fused to allogeneic DC induced lysis of autologous tumor (Fig. 4*B*). Moreover, for both patients, T cells stimulated with OVCA cells fused to autologous or allogeneic DC exhibited induction of CTL activity (Fig. 4*B* and Table I). These findings demonstrate that the antitumor activity of autologous CTLs is stimulated by fusions of tumor cells to autologous or allogeneic DC.

Specificity of OVCA/FC-stimulated CTL

To assess the specificity of CTL induced by fusion cells, T cells stimulated with OVCA cells fused to autologous DC were incubated with autologous tumor, autologous monocytes, MCF-7

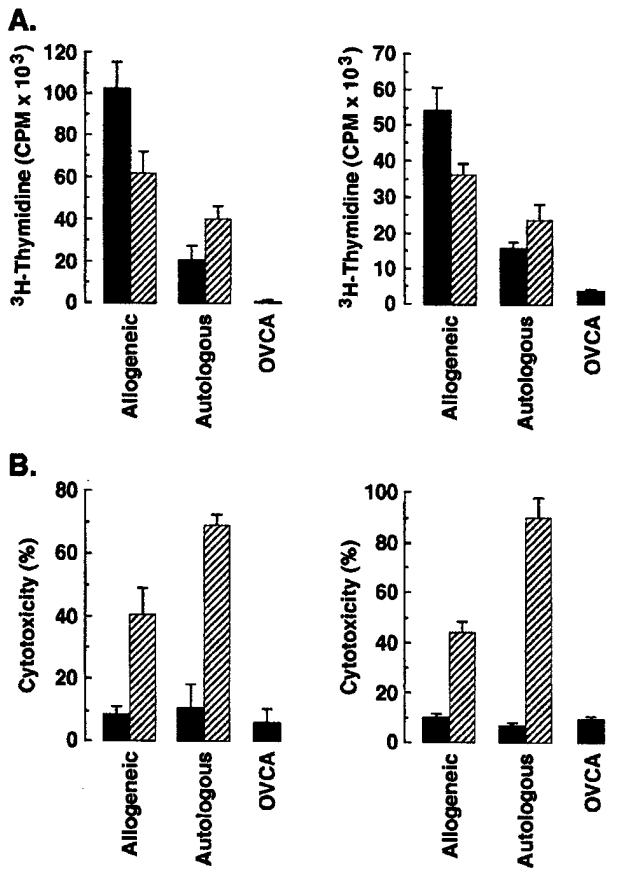

FIGURE 4. Function of antitumor CTL induced by OVCA cells fused to allogeneic and autologous DC. *A*, PBMC were stimulated with the indicated DC (■), OVCA/FC from fusions with allogeneic and autologous DC (▨), or OVCA cells for 5 days in the presence of hIL-2. Uptake of [^{3}H]thymidine was assessed during a 12-h incubation. The results are expressed as the mean ± SD of three replicates. *B*, T cells stimulated with the indicated DC (■), OVCA/FC (▨), or OVCA cells were incubated with ^{51}Cr-labeled autologous OVCA at a ratio of 30:1. Percentage cytotoxicity (mean ± SD of three replicates) was determined by ^{51}Cr release.

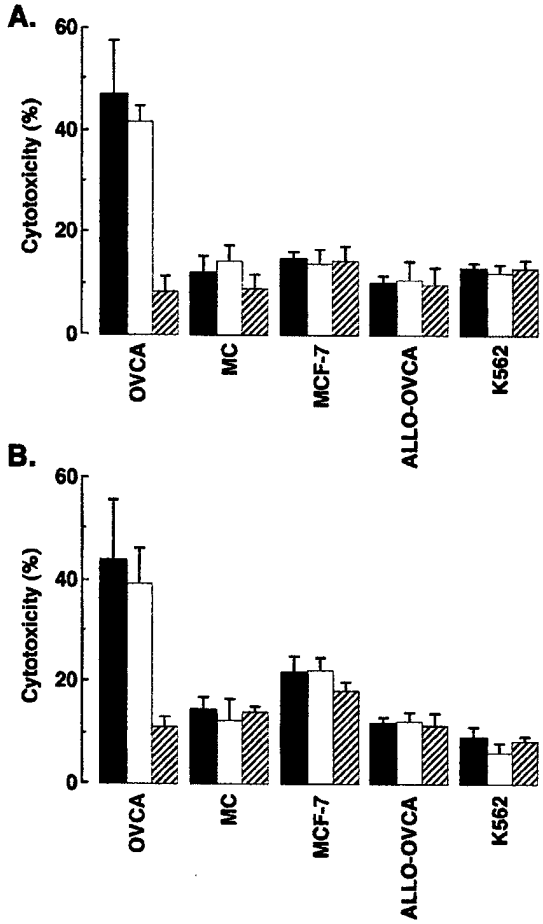

FIGURE 5. Specificity of OVCA/FC-induced CTLs. T cells stimulated with autologous OVCA/FC (*A*) or allogeneic OVCA/FC (*B*) were incubated with ^{51}Cr-labeled autologous OVCA cells, autologous MC, MCF-7 cells, allogeneic OVCA cells (allo-OVCA), or K562 cells at a 30:1 ratio (■). The targets were also preincubated with control IgG (□) or anti-MHC class I Ab (W6/32; 1:100 dilution; ▨) and then assayed for lysis. CTL activity was determined by ^{51}Cr release. The results are expressed as the mean ± SD of three replicates.

breast carcinoma cells, allogeneic OVCA cells, and NK-sensitive K562 cells. Incubation of the OVCA/FC-stimulated T cells with autologous tumor or monocytes demonstrated selective lysis of the tumor (Fig. 5*A*). In addition, there was no significant lysis of the MCF-7, allogeneic OVCA, or K562 cells by these CTL (Fig. 5*A*). Preincubation of the targets with an anti-MHC class I Ab blocked lysis of the autologous OVCA cells and had little effect on that obtained for the other cell types in the absence of Ab (Fig. 5*A*). T cells stimulated with autologous OVCA cells fused to allogeneic

Table I. *Comparison of CTL activity against autologous OVCA cells after incubation of T cells with OVCA, DC, autologous OVCA/FC, or allogeneic OVCA/FC*[a]

		Stimulators				
		Autologous			Allogeneic	
Patient	E:T Ratio	OVCA	DC	OVCA/FC	DC	OVCA/FC
1	30:1	9.2	10.4	68.8	8.51	40.5
	10:1	5.4	8.1	58.9	ND[b]	ND
	3:1	4.8	7.9	15.8	7.0	8.44
2	30:1	9.12	9.95	89.5	9.7	43.9
	10:1	9.02	6.81	70.9	10.1	30.3
	3:1	8.47	4.21	26.6	9.85	9.4

[a] The results represent the percentage lysis (mean of three replicates) of autologous OVCA cells after incubation of autologous T cells with the indicated stimulators.
[b] ND, not done.

DC also demonstrated selective lysis of the autologous tumor (Fig. 5*B*). Moreover, lysis of the autologous tumor was abrogated by preincubation of the targets with anti-MHC class I (Fig. 5*B*).

Discussion

Ovarian carcinoma cells overexpress the CA-125, HER2/neu, and/or MUC1 tumor-associated Ags (11, 13, 15, 17, 42, 43). Little is known about the peptide structure of the CA-125 Ag and, consequently, there is no information available regarding expression of CA-125 peptides that bind to MHC molecules (44, 45). In studies of HER2/neu, multiple HLA-A2.1-binding peptides have been identified that can elicit CTL responses (24, 46, 47). DC from patients with OVCA have been pulsed with synthetic HER2/neu-derived peptides identified by screening the HER2/neu amino acid sequence for HLA-A2.1 anchor residues (24, 47). This approach has defined HER2/neu-derived peptides that induce CTL activity against autologous and allogeneic HLA-$A2^+$ OVCA cells (24). Similar studies have identified HLA-A2-binding peptides from the MUC1 protein (48, 49). DC obtained from normal donors and pulsed with the MUC1 peptides induced CTL, which lyse HLA-A2 tumor cells expressing MUC1 (48).

The present studies describe an alternative approach to a DC-based vaccine for ovarian carcinoma. Previous reports in mouse tumor models have demonstrated that vaccination with heterokaryons prepared by fusing tumor cells and DC induces the regression of established carcinomas, lymphomas, and melanomas (36–39). Fusion cell vaccines have also been shown to be effective in reversing immunologic unresponsiveness to MUC1 in MUC1-transgenic mice (40). In contrast to the identification of tumor-associated peptides that associate with HLA molecules, fusion cells provide a strategy for inducing immunity against both known and unknown tumor Ags (36). In addition, as fusion cells present peptides in the context of MHC class I molecules expressed by the tumor cell, this vaccine is not restricted to certain HLA types. In this regard, fusion of human OVCA cells to DC is associated with expression of the OVCA-associated Ags, CA-125 and MUC1, and DC-derived costimulatory and adhesion molecules necessary for the activation of T cells. The results also demonstrate that the fusion cells are functional in inducing MHC class I-restricted CTL activity.

The demonstration that human OVCA cells can be fused to DC provides the experimental basis for using these fusions as vaccines in the treatment of ovarian cancer. OVCA cell fusions have been generated with both autologous and allogeneic DC. The phenotype of the OVCA cells fused to autologous DC was similar to that obtained with allogeneic DC. In addition, although tumor cells were ineffective in stimulating autologous T cells, fusions of OVCA cells to both types of DC were effective in inducing a T cell proliferative response. As expected, incubation of autologous T cells with OVCA fused to allogeneic DC induced a more pronounced proliferative response than that obtained with OVCA fused to autologous DC. By contrast, induction of CTL activity was similar with OVCA fused to autologous or allogeneic DC. These findings indicate that, although T cell stimulation is greater with OVCA fusions expresssing DC-derived allogeneic Ags, the induction of CTL activity against autologous OVCA cell Ags is not enhanced by fusions to allogeneic, as compared with autologous, DC. Thus, the results support the fusion of OVCA cells to either autologous or allogeneic DC in the development of a clinical vaccine.

OVCA cells fused to either autologous or allogeneic DC were effective in inducing antitumor CTL, which lyse autologous OVCA cells by a MHC class I-restricted mechanism. The autologous OVCA/FC can present tumor Ags by OVCA- or DC-derived MHC class I molecules. Moreover, autologous OVCA/FC can present tumor Ags by DC-derived MHC class II molecules and thereby stimulate helper $CD4^+$ cells. By contrast, presentation of tumor Ags by the allogeneic OVCA/FC cells is dependent on OVCA-derived MHC molecules. The allogeneic OVCA/FC cells can also stimulate alloreactive T cells and thereby the release of cytokines, which contribute to the activation of tumor-specific CTL (50). The induction of antitumor CTL provides an opportunity to define the epitopes, and thereby the tumor-associated Ags, that are targets of the immune response. Thus, although screening of known Ags, such as HER2/neu or MUC1, for epitopes that bind to HLA-A2 represents one approach, the induction of antitumor CTL with fusion cells represents another strategy for defining peptides that function in the immune recognition of tumor cells. The present findings demonstrating human OVCA/DC fusions thus could have potential applicability to the field of antitumor immunotherapy as vaccines and in the identification of novel OVCA-associated Ags.

Note added in proof. Recent studies have demonstrated that fusions of human renal carcinoma cells and allogeneic DC are effective in the treatment of metastatic renal cell cancer (51).

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